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Molecular Cloning of DNA Complementary to mRNA of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus: Location and Gene Products of RNA Transcripts Found Late in Infection†

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DNAs complementary to late *Autographa californica* nuclear polyhedrosis virus (AcNPV) mRNA were synthesized by reverse transcription and cloned in *Escherichia coli* by using pBR322 as a vector. Eleven different cDNAs were distinguished in our screening of 45 AcNPV-homologous clones. Location of the regions of cDNA homology with respect to the AcNPV physical map showed that the 11 cDNAs were dispersed throughout the genome. The most abundant cDNA insertion, representing approximately one-third of the late viral mRNAs, was homologous to the AcNPV *Hin*dIII-P,Q and *Eco*RI-P fragments. The direction of transcription in this region was from left to right on a linearized AcNPV physical map. Hybridization selection followed by in vitro translation showed that this region encoded a 7,200-dalton (7.2K) protein which comigrated with a minor protein found in the extracellular nonoccluded form of the virus (NOV). Similarly, the gene for polyhedrin, the major structural protein of the occluded virus form, was located, at least in part, in the *Hin*dIII-V/*Eco*RI-I region of the AcNPV map. The polyhedrin transcript represented approximately one-quarter of the viral polyadenylic acid-containing RNAs at 27 h postinfection. Another relatively abundant cDNA was homologous to the *Hin*dIII-A/*Eco*RI-C/*Sst*I-G region, and RNA selected by this cDNA directed the synthesis of two proteins (31K and 30K). The protein products of five other cDNA-selected RNAs were identified. The *Hin*dIII-D/*Eco*RI-O, *Hin*dIII-C/*Eco*RI-D, *Hin*dIII-B1/*Eco*RI-E, and *Hin*dIII-B2/*Eco*RI-H regions of the AcNPV L-1 genome were homologous to RNAs which directed the synthesis of a 57K protein, a 25K protein, a 61K protein, and a 37K protein (plus a minor 26K protein), respectively. Late mRNA selected by a cDNA homologous to the *Hin*dIII-P/*Eco*RI-B region of the AcNPV map directed the synthesis of 31K and 30K proteins which comigrated with the 31K and 30K proteins translated from RNA selected by the *Hin*dIII-A/*Eco*RI-C/*Sst*I-G cDNA. Three other cDNAs have not been correlated yet with specific protein products.

One of the most challenging areas of current baculovirology is determining the gene organization of the 128-kilobase (kb) circular, double-stranded DNA genome of the model baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV). One approach to determining gene organization is marker rescue in which genetic mutations are located with respect to the AcNPV restriction fragment physical map by cotransfection of mutant DNA and a wild-type DNA restriction fragment (12). Another approach is to isolate specific fragments of AcNPV DNA, select specific mRNAs by hybridization to the DNA fragment, and determine the protein encoded in the nucleic acid sequences by in vitro translation of the hybrid-selected RNA followed by gel electrophoresis of the resulting protein products. Using two AcNPV genomic fragments as hybridization probes, Vlak et al. have mapped 33,000-dalton (33K) and 39K proteins to the *Eco*RI-I and *Eco*RI-J fragments, respectively (23).

Recent advances in recombinant DNA technology have provided methods for cloning the coding regions of individual virus genes by synthesizing DNA complementary to mRNA, using reverse transcriptase. The use of cDNA clones as hybridization probes for specific mRNAs has distinct advantages over the use of viral genomic

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fragments or fragment clones. Particularly advantageous is that each cDNA selects only a single mRNA or a series of related spliced or symmetrically transcribed RNAs.

In this paper we report the successful synthesis and cloning of DNA complementary to late AcNPV mRNA. Using cDNA clones, we gathered information concerning the relative amounts of various mRNAs found late in infection, the locations of these mRNAs with respect to the physical map of AcNPV L-1 (2, 14), and, in some cases, the identities of the proteins which the mRNAs encode. The physical map location of the 3′ portion of the gene encoding polyhedrin, the major protein of the occluded form of AcNPV, was determined. Information concerning the gene organization of AcNPV should be applicable to both the development of this virus as a vector for genetic engineering (13) and the further development of the virus as a microbial pesticide (20).

MATERIALS AND METHODS

Preparation of RNA from infected cells. Monolayers of *Spodoptera frugiperda* IPLB-SF-21 cells were infected with AcNPV L-1 (9) at a multiplicity of infection of 20. After rocking for 1 h at room temperature, the inoculum was replaced with TC-100 medium, and the cells were incubated at 27°C for 27 h. The cells were suspended, pelleted by centrifugation at 2,000 × *g* and 5°C for 10 min, and washed with phosphate-saline buffer (9). The cells were suspended in phosphate-saline buffer (9) and lysed on ice for 10 min after 0.75% Nonidet P-40 (BDH Chemicals) was added. Nuclei were removed by centrifugation at 4,000 × *g* and 5°C for 10 min. An equal volume of lysis solution (6) was added to the supernatant, and the mixture was extracted twice with phenol (Fluka)-chloroform-isoamyl alcohol (50:50:1) containing 0.1% 8-hydroxyquinoline (6). The RNA was ethanol precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.6) and 2.5 volumes of ethanol and placing the solution at −20°C overnight. After centrifugation at 12,000 × *g* and 4°C for 45 min, the RNA pellet was dissolved in equal parts of phosphate-saline buffer (9) and lysis solution (6), phenol extracted, and ethanol precipitated as described above.

Polyadenylic acid [poly(A)]-containing RNA was isolated by using oligodeoxythymidylic acid cellulose (type 7; P.L. Biochemicals) and a batch adaptation of the procedure of Manley et al. (10). Briefly, 500 μg of cytolasmic RNA in 1 ml of binding buffer (10 mM Tris, pH 7.6, 0.5 M NaCl, 0.2% sodium dodecyl sulfate [SDS]) was added to 50 mg of oligodeoxythymidylic acid cellulose (P.L. Biochemicals) in a microfuge tube and mixed gently for 90 min at room temperature. This was followed by centrifugation at 12,000 × *g* for 3 min and then by three washes with binding buffer; poly(A)-containing RNA was removed by washing with 1 ml of elution buffer (5 mM Tris, pH 7.6, 2 mM EDTA) for 15 min. Elution was repeated with a second 1-ml volume of elution buffer, and the two eluates were pooled. Fine cellulose particles in the resulting preparation were removed by passing the mixture through siliconized glass wool in a Pasteur pipette, and the RNA was ethanol precipitated.

Synthesis of cDNA from late mRNA. Double-stranded cDNA was prepared from poly(A)-containing RNA by the procedure of Wickens et al. (24). The 100-μl reaction mixture contained poly(A)-containing RNA derived from 500 μg of cytoplasmic RNA, 10 μg of oligodeoxythymidylic acid$_{12-18}$ (P.L. Biochemicals), and each deoxynucleoside triphosphate at a concentration of 500 μM, including [α-^{32}P]dCTP (2 Ci/mmol; New England Nuclear Corp.) in 50 mM Tris-hydrochloride (pH 8.3)–140 mM KCl–30 mM 2-mercaptoethanol–10 mM $MgCl_2$. Reaction mixtures were assembled on ice, 50 U of reverse transcriptase (J. Beard, Life Sciences, Inc.) was added, and the reaction mixtures were mixed, centrifuged briefly, and placed at 42°C for 60 min. For second-strand synthesis, the first-strand reaction mixture was heated for 3 min at 100°C, cooled in an ice water bath, centrifuged for several seconds, and transferred to a solution consisting of 50 μl of each deoxynucleoside triphosphate (concentration, 1 mM) and 50 μl of 400 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.9). Then 20 U of *Escherichia coli* DNA polymerase I was added, and the reaction mixture was incubated for 2 h at 15°C.

Reactions were stopped by adding 2.5 mM dGTP, 0.03% SDS, and 18 μg of *E. coli* soluble RNA (Calbiochem) and extracting with phenol twice. The aqueous phase was passed through a Sephadex G-50 column, and the excluded volume was ethanol precipitated.

The hairpin loop in the cDNA was cut by S1 nuclease digestion in a solution containing 250 mM sodium chloride, 30 mM sodium acetate, 1 mM zinc sulfate, and 5% glycerol (pH 4.6). After digestion with 125 U of S1 nuclease (Sigma Chemical Co.) for 60 min at 37°C, the mixture was phenol extracted, ether extracted, and ethanol precipitated. The final yield was 400 ng of double-stranded cDNA.

Construction of hybrid plasmids. Homopolymer tailing of the cDNA was accomplished by using the method of Roychoudury and Wu (17). We used 50 ng of cDNA and 25 U of terminal transferase (Bethesda Research Laboratories, Inc.) in a solution containing 100 mM potassium cacodylate, 1 mM $CoCl_2$, 200 μM dithiothreitol, and 10 μM dCTP (pH 7.0). The reaction mixture was incubated for 30 min at 37°C, and the DNA was ethanol precipitated. Purified plasmid pBR322 was digested with *Pst*I (Bethesda Research Laboratories, Inc.) phenol extracted, ether extracted, and ethanol precipitated. Approximately 1 μg was tailed with deoxyguanine by using 10 μM dGTP and 60 U of terminal transferase. The guanine-tailed vector DNA was then ethanol precipitated and dissolved in 1 ml of 10 mM Tris-hydrochloride (pH 8)–10 mM EDTA–100 mM NaCl. The vector DNA was added to 50 ng of cytosine-tailed cDNA in 10 μl of 10 mM Tris (pH 7.6)–1 mM EDTA. The mixture of DNAs was heated for 10 min in a 65°C water bath. Then the bath was shut off and allowed to cool to room temperature overnight for annealing of the guanine and cytosine tails of the DNAs. *E. coli* soluble RNA (20 μg) was added, and the DNA was precipitated. This chimeric DNA was used to transform *E. coli* RR1 (7), and colonies were selected by resistance to tetracycline and sensitivity to ampicillin. P1 physical containment

was used, as required by the National Institutes of Health guidelines for recombinant DNA research.

Screening recombinant plasmids. Bacterial colonies with the appropriate drug responses were grown overnight on LB agar (1% tryptone [Difco Laboratories], 1% NaCl, 0.5% yeast extract, 0.1% glucose) overlaid with nitrocellulose filters (type BA85; Schleicher & Scheull Co.), and each filter was transferred to an LB agar plate containing 170 μg of chloramphenicol per ml and incubated overnight. The filters were processed on pads of saturated filter paper as described previously (3), with a final soak on a filter pad saturated with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Excess liquid was removed by suction, and each nitrocellulose filter was placed on a 95% ethanol pad, dried by suction, and baked in a vacuum oven at 80°C for 2 h. Colonies containing AcNPV insertions were recognized by hybridization with an AcNPV DNA probe labeled with ^{32}P by nick translation (see below).

Labeling of DNA by nick translation. AcNPV DNA or plasmid DNA was labeled in vitro by a nick-translation procedure (16), using 25 μCi of [$\alpha^{32}P$]dCTP (800 Ci/mmol in 0.01 M Tricine, pH 7.6; New England Nuclear Corp.). After 2 h at 15°C, the 25-μl reaction volume was brought to 200 μl with 10 mM Tris (pH 7.6)–1 mM EDTA and phenol extracted twice. Calf thymus DNA (100 μg) was added, and the DNA was ethanol precipitated by placing the preparation in dry ice for 20 min. Two more ethanol precipitations were done to remove unincorporated dCTP before use in hybridization experiments.

DNA hybridization conditions. Filters were preincubated in a solution containing 0.02% bovine serum albumin and 0.02% Ficoll 400 in 3× SSC in boilable bags at 65°C for 4 h. This solution was replaced with a hybridization solution containing 50% formamide (Fluka), 5× SSC, 10 mM HEPES (pH 6.9), 0.1% SDS, 1 mM EDTA, 0.02% bovine serum albumin, and 0.02% Ficoll 400. Probe DNA in 5× SSC was denatured at 100°C for 10 min and added to each bag. Incubation was for 20 h at 37°C with gentle shaking. Filters were washed three times (45 min each time) in 5× SSC containing 0.2% SDS at 65°C, and this was followed by a 2-h wash in 2× SSC at room temperature. The filters were then exposed to preflashed Kodak X-ray film for different times at −70°C, using Cronex Lightning Plus intensifying screens.

Electrophoresis of DNA. AcNPV DNA was digested with either *Hin*dIII, *Eco*RI, or *Sst*I according to the directions of the manufacturer (Bethesda Research Laboratories, Inc.). Electrophoresis was carried out at 75 mA for 20 h in 0.7% agarose gels submerged in 40 mM Tris-acetate (pH 7.8)–5 mM sodium acetate–1 mM EDTA (TAE buffer) (21) containing 0.5 μg of ethidium bromide per ml. DNA was transferred from agarose gels to nitrocellulose (a Southern blot method) by the bidirectional transfer technique (19).

Recombinant cDNA plasmid DNA (2 μg) was digested with *Pst*I and subjected to electrophoresis at 120 mA for 15 h through 1.4% agarose in TAE buffer supplemented with 0.5 μg of ethidium bromide per ml.

Hybridization selection of mRNA. Specific RNAs were selected from a mixture of cytoplasmic RNAs isolated from AcNPV-infected *S. frugiperda* cells by hybridization to recombinant plasmid cDNA bound to nitrocellulose, using a composite of the procedures described by Ricciardi et al. (15) and Vlak et al. (23). Recombinant plasmid DNAs (50 μg) in 10 mM Tris (pH 7.6)–1 mM EDTA were boiled for 30 min, brought to 5× SSC, and passed slowly through nitrocellulose filters (diameter, 24 mm) prewet with 5× SSC. The DNA-containing filters were dried and then baked in vacuo at 80°C for 2 h. The filters were cut into small pieces and presoaked in hybridization buffer containing 50% formamide, 600 mM NaCl, 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8), 4 mM EDTA, and 0.5% SDS supplemented with 50 μg of poly(A) (Sigma Chemical Co.) per ml for 1 to 2 h at 37°C. The hybridization buffer was removed and replaced with 200 μg of cytoplasmic RNA in 200 μl of hybridization buffer. Hybridization was for 8 h at 37°C and was followed by washing and elution (23); 0.1 volume of 20% potassium acetate and 25 volumes of ethanol were added to the eluted RNA, and the preparation was stored at −20°C. The filters were regenerated by soaking for 20 min in 0.1 N NaOH in 2× SSC, followed by washing five times with 2× SSC and drying in a vacuum desiccator for 2 h (11).

In vitro translation of RNA. Cytoplasmic RNA, which was stored as a precipitate in sodium acetate-ethanol at −20°C, was centrifuged, and the resulting pellet was dissolved in 2% potassium acetate and precipitated with ethanol. For cell-free translations, RNAs were centrifuged, washed in 70% ethanol, dissolved in water, and lyophilized. The rabbit reticulocyte lysate system of Bethesda Research Laboratories, Inc. was used according to the recommended protocol. L-[3,4,5-^3H]leucine (110 Ci/mmol; New England Nuclear Corp.), which was supplied in 0.01 N HCl, was neutralized with 0.10 volume of 0.1 N KOH before use. Each 30-μl assay mixture contained 4 μCi of [^3H]leucine and final concentrations of 154 mM K^+ and 1.2 mM Mg^{2+}. Reaction mixtures were incubated at 30°C for 60 min.

SDS gel electrophoresis. The electrophoretic procedure of Laemmli (8) was slightly modified for our analysis of proteins. The modifications included the use of 0.75 M Tris (pH 8.8) instead of 0.375 M Tris (pH 8.8) in the separating gel and the use of 0.25 M Tris (pH 6.8) instead of 0.125 M Tris (pH 6.8) in the stacking gel. Stacking gels were 1 cm tall and contained 3.6% acrylamide. Separating gels contained 10% acrylamide and were 1.5 mm thick by 17 cm tall. Electrophoresis was at 95 V for 15 h, during which time the tracking dye migrated approximately 16 cm. Gels were fixed in methanol-water-acetic acid (5:5:1) for 1 h, impregnated with En^{3}Hance (New England Nuclear Corp.) for 1 h, soaked in water for 1 h, and dried. Fluorography was done by exposing preflashed Kodak XAR film to gels at −70°C, using Du Pont Cronex intensifying screens.

For the analysis of proteins having molecular weights less than 14,000, we used a procedure adapted from Shapiro et al. (18) by Bethesda Research Laboratories, Inc. The resolving gel (17 cm by 1.5 mm) of 15% polyacrylamide (ratio of bisacrylamide to acrylamide, 0.8:30) contained 0.1 M sodium phosphate (pH 7.2), 0.1% SDS, and 6 M urea. The upper gel contained 3.5% acrylamide in the same buffer as the resolving gel. Only 2 mm of upper gel was between the well bottoms and resolving gel. The running buffer was 0.1 M sodium phosphate (pH 7.2) supplemented with 0.1% SDS and was recirculated during electrophoresis.

Protein samples were brought to 10 mM sodium phosphate (pH 7.2), 7 M urea, 1% SDS, 1% 2-mercaptoethanol, and 0.01% bromphenol blue. The samples were heated for 2 min at 100°C before they were loaded onto the gel. Electrophoresis was at 95 V for 19 h, and this was followed by fixing for 1 h in 15% isopropanol–10% acetic acid. Gels were soaked for 2 h in En^{3}Hance (New England Nuclear Corp.), processed, and exposed to film as described above. For protein standards the high- and low-molecular-weight ^{14}C-labeled protein standards of Bethesda Research Laboratories were used.

RESULTS

Location and frequency of cDNA clones of late AcNPV RNA. DNA complementary to late AcNPV mRNA was cloned in *E. coli*, using pBR322 as a vector (see above). Starting with 500 μg of cytoplasmic RNA, we obtained 0.4 μg of double-stranded cDNA, 50 ng of which was annealed to pBR322 via homopolymer tails, and the recombinant DNA was used to transform *E. coli* RR1. The yield was approximately 1,400 ampicillin-sensitive, tetracyline-resistant colonies. Approximately 20% of these colonies contained AcNPV sequences, as shown by colony hybridization when AcNPV DNA was used as a probe.

A total of 45 AcNPV-homologous cDNA clones were located on the AcNPV physical map by annealing ^{32}P-labeled, nick-translated recombinant plasmid DNAs to Southern blots of *Hin*dIII, *Eco*RI, and *Sst*I enzyme digests of AcNPV DNA. Figure 1 shows an autoradiogram of AcNPV *Hin*dIII and *Eco*RI blots probed with [^{32}P]DNAs isolated from 10 different cDNA clones. These clones were designated pMA, referring to plasmids cloned by one of us (M.J.A.), followed by letters to designate the AcNPV *Hin*dIII, *Eco*RI, and, if necessary, *Sst*I fragments to which they were homologous. For instance, a cDNA that hybridized to AcNPV *Hin*dIII-V and *Eco*RI-I was designated pMA-VI. All hybridization locations were consistent with the physical map of AcNPV L-1 DNA shown in

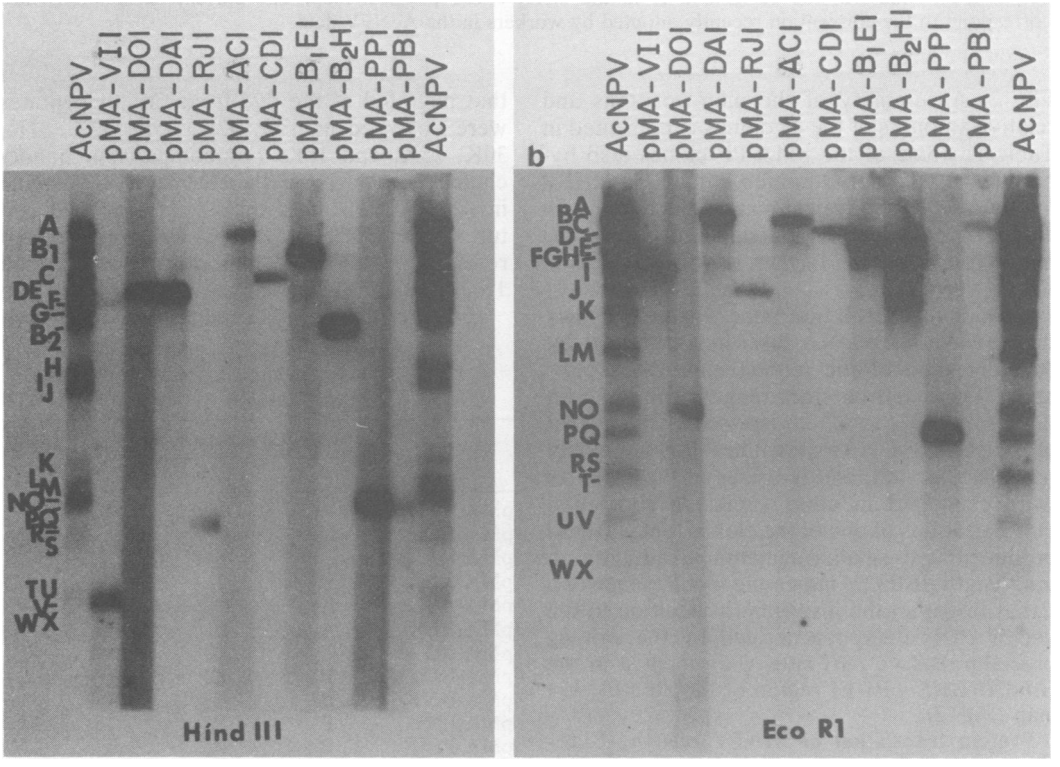

FIG. 1. Hybridization of ^{32}P-labeled AcNPV cDNA plasmids to *Hin*dIII-digested (a) or *Eco*RI-digested (b) AcNPV DNA blots. A 0.7% agarose slab gel containing fractionated *Hin*dIII- or *Eco*RI-digested AcNPV DNA was blotted onto nitrocellulose filter paper. Strips of the resulting blots were hybridized to ^{32}P-labeled cDNA plasmids or ^{32}P-labeled AcNPV DNA as a control. After hybridization, the blots were autoradiographed. The positions of fragments on the blots are shown on the left of each blot; the letters correspond to the recently accepted convention for AcNPV fragment designations. The first and last strips of each blot were hybridized to the entire AcNPV L-1 DNA. Other strips were hybridized to the clones designated.

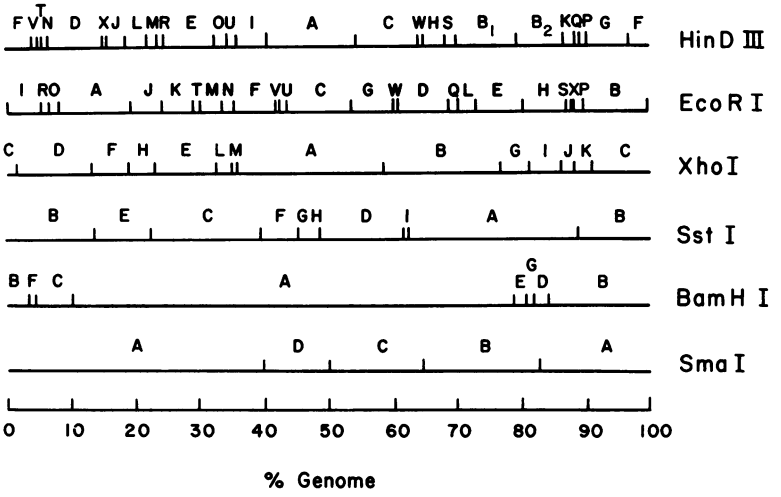

FIG. 2. Physical map of the L-1 variant of AcNPV. The circular map of AcNPV was linearized for ease of presentation. The restriction map sites are a combination of the sites determined by Miller and Dawes for L-1 (14) and the sites determined by Cochran et al. for HR (2). The fragment letter designations are similar to those described by Cochran et al. (14), except that the *Hin*dIII fragment in the 40- to 50-map unit region is designated *Hin*dIII-A and the two *Hin*dIII fragments in the 70- to 85-map unit region are designated B1 and B2. The assignment of B1 and B2 to specific AcNPV L-1 *Hin*dIII fragments is shown in Fig. 1. Formerly (14), B1 was designated B and B2 was designated H in the AcNPV L-1 physical map. The current designations now correspond to the convention recently adopted by workers in the AcNPV field.

Fig. 2. A summary of the map positions and relative amounts of the 45 clones is presented in Table 1. Some of the pMA-PP clones also hybridized to *Hin*dIII-Q, which adjoins *Hin*dIII-P on the AcNPV physical map. When multiple clones were available for a single map position, one was chosen for further study (e.g., pMA-VI1).

Sizes of the cDNA insertions. Figure 3 shows the sizes of the cDNA insertions obtained by *Pst*I digestion of the recombinant DNA plasmids. The insertion sizes ranged from 0.22 kb (for pMA-DO1) to 1.5 kb (for pMA-DA1). Two of the plasmid DNAs shown in Fig. 3 were only partially digested, and the sizes of these DNAs were estimated in other similar experiments. *Pst*I digestions of one of the clones (pMA-B1E1) produced two small fragments in addition to unit-length pBR322, indicating the presence of a *Pst*I site within the insertion (in addition to the vector *Pst*I site[s] regenerated by the cloning procedure). Two *Pst*I sites were located in the *Hin*dIII-B1/*Eco*RI-EI region of the AcNPV L-1 map (Fig. 2).

Protein translation of AcNPV mRNA. Cytoplasmic RNA that was prepared from cells 27 h after infection with AcNPV was translated in vitro by using a rabbit reticulocyte lysate system. The [^{3}H]leucine-labeled proteins were separated on 10% polyacrylamide gels and autoradiographed (Fig. 4, lanes b and f). We observed at least 25 peptides with molecular weights between 85,000 and 16,000 and additional peptides that migrated at the dye front. Major peptides were observed at 63K, 42K, 35K, 32K, 31K, 30K, 25K, and 18K. For comparison, nonoccluded virions (NOV) labeled with [^{3}H]leucine in AcNPV L-1-infected *S. frugiperda* cell cultures were purified (9), and 21 proteins were resolved on 10% SDS gels (Fig. 4, lane a) and 15% SDS–urea gels (see below).

Hybridization selection and protein translation.

TABLE 1. Locations and abundance of cDNA clones

Clone	Physical map positions			No. of clones[a]
	*Hin*dIII	*Eco*RI	*Sst*I	
pMA-VI[b]	V	I		12
pMA-DO	D	O		1
pMA-DA	D	A		1
pMA-RJ	R	J		1
pMA-ACG	A	G	G	4
pMA-ACD	A	C	D	2
pMA-CD	C	D		2
pMA-B1E	B1	E		1
pMA-B2H	B2	H		3
pMA-PP	P	P		17
pMA-PB	P	B		1

[a] A total of 45 AcNPV-homologous cDNA clones were located on the AcNPV L-1 physical map.

[b] All cDNA plasmids are designated pMA, indicating that one of us (M.J.A.) constructed the clones, and then distinguished by letters indicating their *Hin*dIII, *Eco*RI, and, in some cases, *Sst*I map positions. For instance, the first clone listed is homologous to *Hin*dIII-V and *Eco*RI-I fragments of AcNPV L-1.

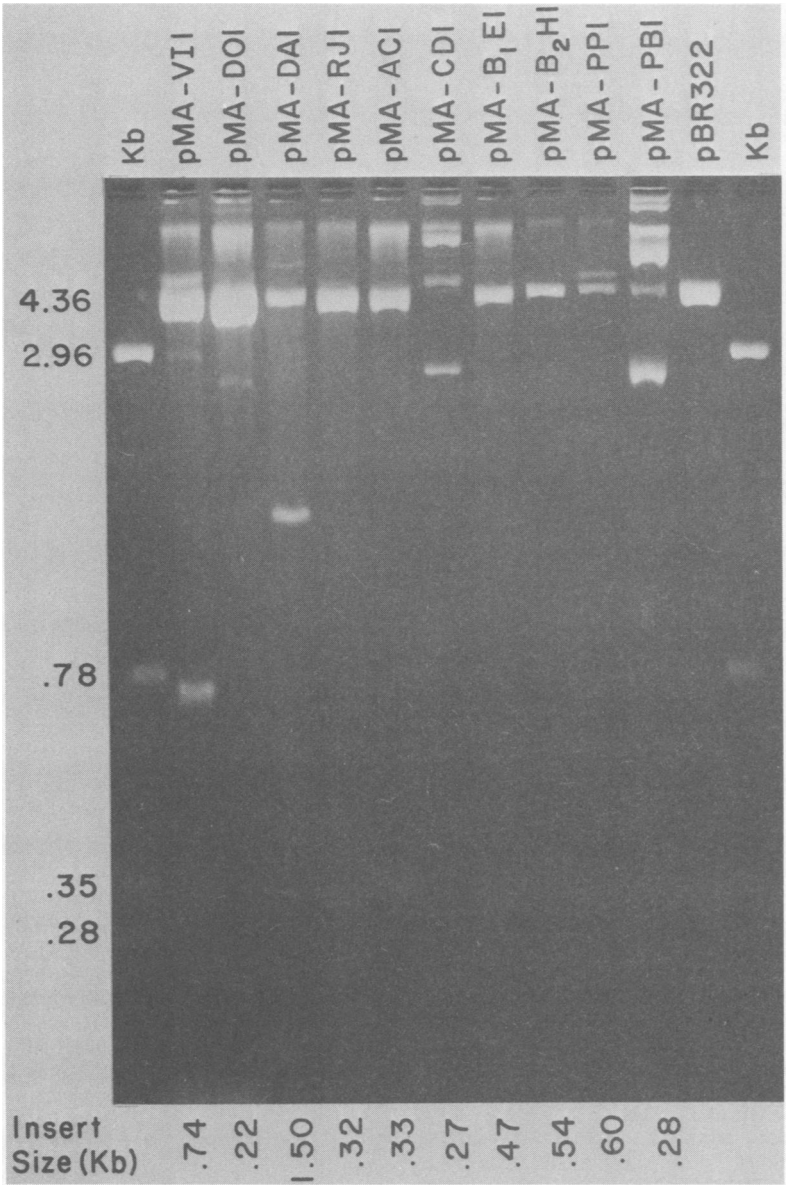

FIG. 3. *Pst*I fragments of AcNPV cDNA-containing plasmids. Samples (2 μg) of plasmid DNA were digested with *Pst*I and loaded into slots of a 1.4% agarose horizontal slab gel. As molecular weight markers, pBR322 was codigested with *Hin*dIII, *Bam*HI, *Sal*I, and *Pst*I and loaded into the outer slots. cDNA clones are indicated at the top, and insertion sizes are indicated at the bottom in kilobase pairs (Kb). Digestion was not complete for pMA-CD1 and pMA-PB1, and the sizes of these clones were determined in a similar experiment.

RNAs homologous to the cloned cDNA plasmid DNAs were purified from total cytoplasmic RNA isolated from *S. frugiperda* cells late (27 h) in infection by hybridization to the plasmid cDNAs bound to nitrocellulose. The cDNA-homologous RNAs were eluted and then translated into proteins in vitro.

As shown in Table 1, 17 clones were isolated from the *Hin*dIII-P/*Eco*RI-P region, 12 clones were isolated from the *Hin*dIII-V/*Eco*RI-I region, and 4 clones were isolated from the *Hin*dIII-A/*Eco*RI-C/*Sst*I-G region of the AcNPV genome. Hybrid selections and translations with representatives of these clones gave 10-, 4-, and 3-fold stimulation, respectively, compared with endogenous protein synthesis levels of the lysate system, suggesting that the frequency of isolates from each region correlated with the amounts of

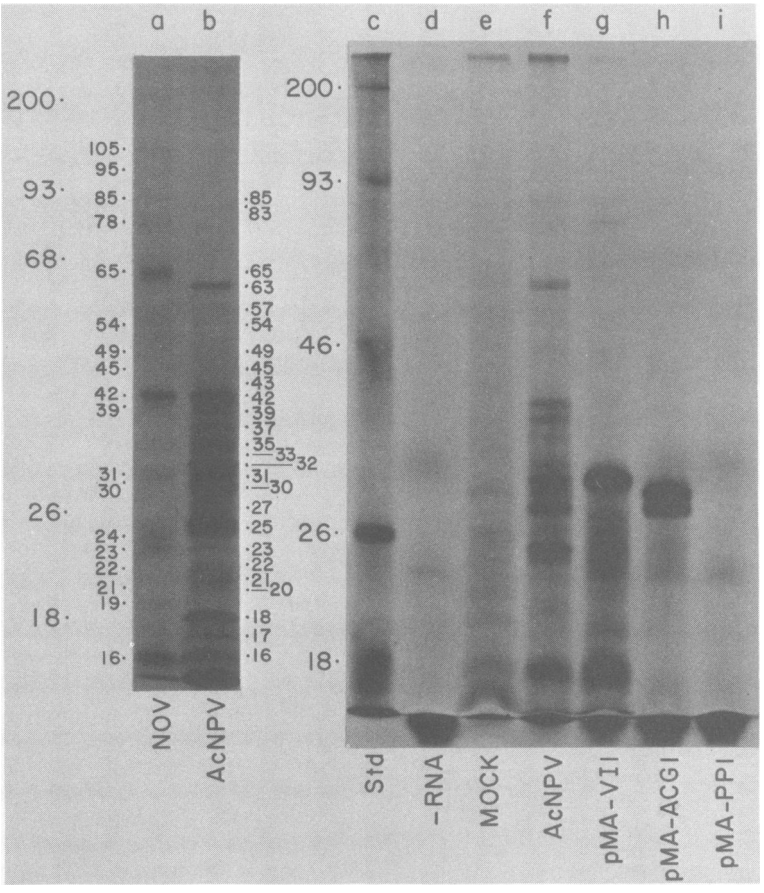

FIG. 4. SDS-polyacrylamide gel fluorogram of in vitro translation products of AcNPV-infected cell RNA and AcNPV cDNA-specific RNA. For comparison, [^{3}H]leucine-labeled NOV (lane a) were included; NOV peptide molecular weights ($\times 10^3$) are indicated on the left. Lane b shows the results of translation of AcNPV-infected cell RNA (25 μg) in a cell-free rabbit reticulocyte lysate system, and the corresponding peptide molecular weights ($\times 10^3$) are indicated on the right. Lane d, Translation products without added RNA; lanes e through i, translation of RNA from mock-infected cells, RNA from AcNPV-infected cells, pMA-VI1-selected RNA, pMA-ACG1-selected RNA, and pMA-PP1-selected RNA, respectively. Lanes a and b and lanes c through i are from two separate 10% SDS–polyacrylamide gels. Gels impregnated with En^{3}Hance (New England Nuclear Corp.) were exposed to film for 7 days. The molecular weights ($\times 10^3$) of ^{14}C-labeled protein standards (std) (lane c) are indicated on the left of each gel.

the respective mRNAs in the cytoplasmic pool. Less than twofold stimulation was observed for those clones for which only one to three clones were isolated. The ^{3}H-labeled proteins synthesized from the selected mRNAs were resolved on 10% SDS gels (Fig. 4 and 5). The pMA-VI1-selected RNA encoded a major 32K protein (Fig. 4, lane g) that migrated at the position of polyhedrin, the major structural protein of the occluded form of AcNPV (see below). In addition to the 32K protein, we observed a faint background of peptides and a major 18K peptide.

Two proteins (31K and 30K) were synthesized in approximately equal quantities from pMA-ACG1-selected RNA (Fig. 4, lane h). Both of these proteins comigrated with NOV structural proteins (compare Fig. 4, lanes a and h).

Proteins synthesized from pMA-PPI moved with the dye front (Fig. 4, lane i) and were resolved by electrophoresis in 15% SDS–urea gels (Fig. 6). In these gels, which were specifically designed for accurate analysis of the sizes of low-molecular-weight polypeptides, the major polypeptide directed by pMA-PP1-selected RNA was a 7.2K protein that comigrated with a faint NOV structural protein (Fig. 6, lanes c and d). The larger 19K and 23K proteins in Fig. 6, lane d, corresponded to protein products synthesized from endogenous mRNA in the lysate system (Fig. 6, lane b).

Longer exposure times were required to re-

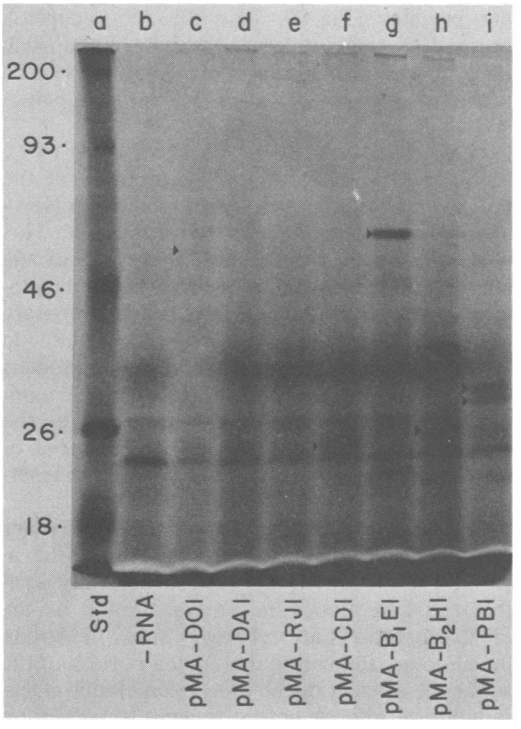

FIG. 5. In vitro translation products of additional AcNPV cDNA-specific RNAs. Lane a contained ^{14}C-labeled protein standards (std); the molecular weights of these standards ($\times 10^3$) are indicated on the left. Lane b shows the translation products when no RNA was added. The peptide from pMA-DO1-specific RNA (lane c) is indicated by an arrowhead. pMA-DA1 (lane d) and pMA-RJ1 (lane e) did not direct the synthesis of detectable peptides. The translation products from specific RNAs for pMA-CD1 (lane f), pMA-B1E1 (lane g), pMA-B2H1 (lane h), and pMA-PB1 (lane i) are indicated by arrowheads. The gel was 10% SDS–polyacrylamide, and fluorographic exposure was for 26 days.

veal the proteins encoded by the remaining cDNA-selected RNAs. A 56K protein was synthesized from pMA-DO1-selected RNA (Fig. 5, lane c), and this protein did not appear to comigrate with any NOV structural protein. Under the conditions used, no proteins were observed as products of in vitro translation of RNA selected by either pMA-DA1 or pMA-RJ1. pMA-ACD1 was not tested in the in vitro translation assay.

RNA selected by pMA-CD1 directed the synthesis of a 25K protein which comigrated with a structural NOV protein. A 60K protein was synthesized from pMA-B1E1-selected RNA, and this protein did not correspond to viral structural proteins.

pMA-A2H-selected RNA directed the synthesis of a 37K protein and, to a lesser extent, a 26K protein. Neither of these proteins appeared to correspond to viral structural proteins. The RNA selected by pMA-PB1 directed the synthesis of two proteins (31K and 30K) in approximately equal quantities. These proteins comigrated with NOV structural proteins and with the proteins synthesized from pMA-ACG1 (Fig. 4, lane h). A weak band at 50K was also observed in Fig. 5, lane i, and may represent an additional protein product.

pMA-VI1 corresponds to the polyhedrin gene. To demonstrate that the 32K protein synthesized from pMA-VI1-selected RNA was polyhedrin, we performed immune precipitations (5) with antisera raised to purified polyhedrin (Fig. 7). Immune precipitation of in vitro-synthesized

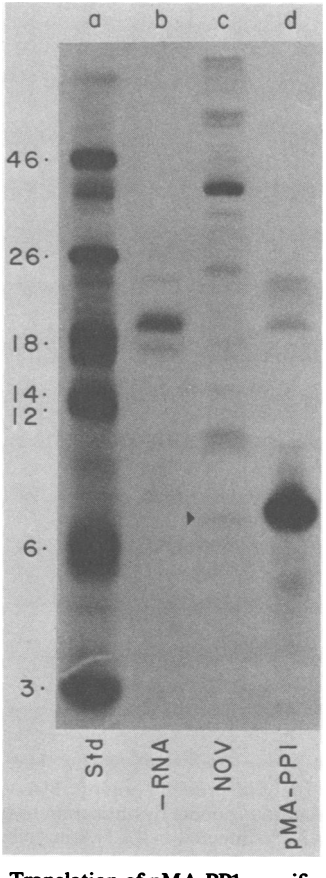

FIG. 6. Translation of pMA-PP1-specific RNA and peptide separation on SDS–urea–15% polyacrylamide gels. Lane a contained ^{14}C-labeled protein standards (std); the molecular weights ($\times 10^3$) of these standards are indicated on the left. Lane b contained cell-free rabbit reticulocyte translation products with no added RNA. For comparison, lane c contained NOV with the 7.2K peptide (arrowhead). Lane d contained translation products of pMA-PP1-specific RNA. Fluorography was for 5 days.

proteins directed by total RNA isolated late in AcNPV-infected *S. frugiperda* cells included a major 32K protein that comigrated with purified polyhedrin, an 18K protein, and a few faint polypeptides with molecular weights between 18,000 and 32,000 (Fig. 7, lanes a and b). These immunoprecipitated products corresponded in size and relative intensity to the peptides synthesized from pMA-VI1-selected RNA (Fig. 7, lane c). Immune precipitation of the protein products of pMA-VI1-selected RNA resulted in the precipitation of the 32K, 18K, and faint intermediate proteins (Fig. 7, lane d). As a control, purified [^{3}H]leucine-labeled polyhedrin (Fig. 7, lane e) was also immune precipitated (Fig. 7, lane f).

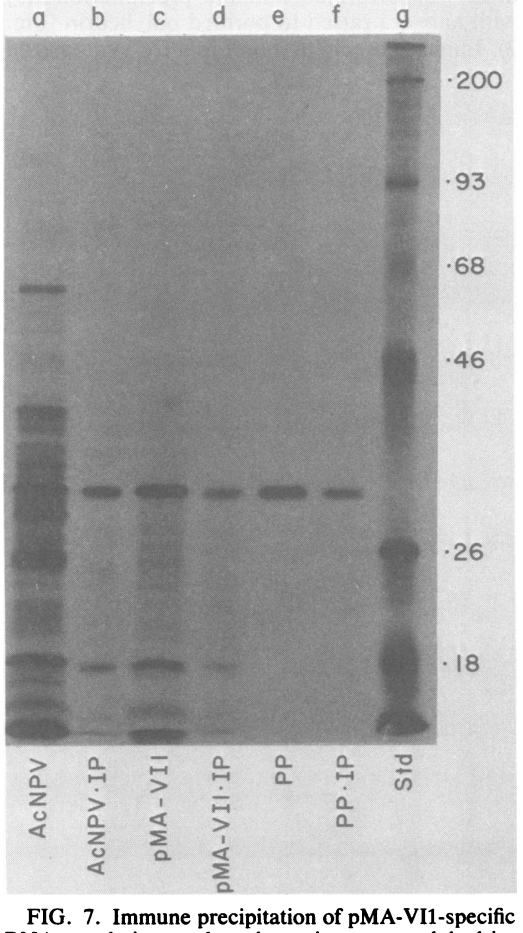

FIG. 7. Immune precipitation of pMA-VI1-specific RNA translation products by antiserum to polyhedrin. Lane a, AcNPV-infected cell RNA in cell-free rabbit reticulocyte lysate translation reaction; lane b, immune precipitation of the proteins in lane a by antiserum to polyhedrin; lane c, translation of pMA-VI1-specific RNA; lane d, precipitation with polyhedrin antiserum; lane e, [^{3}H]leucine-labeled purified polyhedrin; lane f, precipitation of ^{3}H-labeled polyhedrin with antiserum to polyhedrin. Lane g contained ^{14}C-labeled protein standards (std); the molecular weights ($\times 10^3$) of these standards are indicated on the right. The gel was a 10% SDS–polyacrylamide gel, and fluorography was for 7 days.

DISCUSSION

By 27 h postinfection, 20% or more of the poly(A)-containing mRNA found in AcNPV-infected *S. frugiperda* cells is virus specific. This is demonstrated by the fact that 20% of the ampicillin-sensitive, tetracycline-resistant colonies, obtained by cloning DNA complementary to poly(A)-containing RNA isolated late in AcNPV infection, contain AcNPV-homologous sequences. The value 20% represents a minimum since some AcNPV insertions may be too small to respond visibly to the AcNPV probe or may be ampicillin sensitive but contain no insertion.

A total of 11 different DNAs complementary to poly(A)-containing RNAs present late in AcNPV infection have been successfully synthesized and cloned in *E. coli*. Many of the cDNA insertions are relatively small (less than 0.5 kb long), indicating that only a portion of the sequence of each mRNA has been cloned. Degradation of mRNA before reverse transcription does not appear to be a problem since in vitro translation of the same RNA results in proteins having molecular weights up to 85,000. Oligodeoxythymidylic acid was used as a primer for reverse transcription, and it is therefore probable that only the 3′ ends of the mRNAs were cloned. Each cDNA insertion provides a valuable tool for exploring the gene organization and expression of AcNPV.

The frequencies of appearance of the different cDNA sequences (Table 1) may correlate with the relative amounts of the sequences in the mRNA population. The most abundant cDNA is represented by pMA-PP1 and may represent more than one-third of the AcNPV-specific poly(A)-containing RNA. A very small protein (7.2K) is translated from this mRNA. A protein of similar size is found as a minor component of extracellular NOV. The 7.2K protein does not correspond in size to the protamine-like protein found in AcNPV nucleocapsids (22). The direction of transcription of the RNA in the *Hin*dIII-P/*Eco*RI-P region is probably from left to right on the physical map (Fig. 2) since the smaller pMA-PP clones hybridize only to *Hin*dIII-P and the larger clones hybridize to both *Hin*dIII-P and -Q. The transcript must lie on the left side of *Hin*dIII-P since another clone, pMA-PB1, hybridizes to *Hin*dIII-P and *Eco*RI-B (Fig. 2).

The second most abundant cDNA found at 27 h postinfection is homologous to the mRNA that directs the synthesis of polyhedrin, a 32K protein which is the major structural protein of the

occluded form of AcNPV. Vlak et al. (23) have previously implicated *Eco*RI-I as the location of the polyhedrin gene by hybridization selection with *Eco*RI-I isolated from gels of viral DNA fragments, followed by in vitro translation of the selected RNA. We have further pursued the location of polyhedrin by defining *Hin*dIII-V as the probable 3′ end of the polyhedrin message and by demonstrating that the 32K protein product, which is translated from pMA-VI1-selected mRNA, not only comigrates with purified polyhedrin but also immunoprecipitates specifically with antisera to purified polyhedrin.

The pMA-VI1 cDNA insertion also directs the synthesis of a prominent 18K protein, as well as a few fainter bands (Fig. 7). These proteins are antigenically related to polyhedrin because they also specifically immunoprecipitate with polyhedrin antibody. There are several possible explanations for the presence of the 18K protein. The first involves splicing of AcNPV mRNA such that two different transcripts contain the same 3′ terminus but encode different proteins. This explanation is unlikely in light of recent transcriptional studies in our laboratory in which pMA-VI1 DNA was used as a probe of Northern RNA blots; this work indicated that pMA-VI1 hybridizes to a prominent 1.2-kb RNA (D. W. Miller, and L. K. Miller, manuscript in preparation). Although we have not excluded the possibility of splicing at the 5′ end of the polyhedrin mRNA, the evidence suggests that there is only one major distinct mRNA homologous to pMA-VI1 (the 3′ end). Furthermore, a major 18K protein has not been observed in [^{35}S]methionine-labeled infected cells at 27 h postinfection (L. K. Miller and R. Trimarchi, unpublished data), which argues against the possibility that two different proteins are made from the same mRNA. Based on these considerations, we currently favor the explanation that the 18K protein and the other faint proteins antigenically related to polyhedrin are prematurely terminated polypeptides (i.e., artifacts of the in vitro translation system). Specific prematurely terminated translation products have been observed by a number of other workers who used in vitro translation systems and may be due to a deficiency of some tRNA species in the translation system.

Another abundant insertion found at 27 h postinfection is represented by pMA-ACG1. This clone is interesting because it selects an RNA(s) that encodes two proteins (a 31K protein and a 30K protein), which are synthesized in approximately equal quantities. We favor the explanation of spliced RNA in this case since there are two predominant 31K and 30K proteins found in infected cells late in AcNPV infection, as well as two NOV 31K and 30K structural proteins. Furthermore, hybridization of pMA-ACG1 to Northern blots of late RNA has indicated the presence of two predominant RNA species, which are approximately 1.1 and 1.3 kb long (Miller and Miller, unpublished data). The use of cDNA clones rather than fragments of viral genomic DNA is clearly advantageous in this work.

An unusual observation is that pMA-PB1-selected RNA also directs the synthesis of approximately equal quantities of two proteins (31 K and 30K proteins) which comigrate with the pMA-ACG1-directed proteins. The pMA-ACG and pMA-PB DNAs hybridize to regions at map positions 43 to 50 and 89.5 to 90.5 on the AcNPV physical map, respectively (almost diametrically opposed on a circular map), and there is no apparent cross-hybridization between these two regions, even when the blots are overexposed. The pMA-PB1 insertion reproducibly selects for RNAs that direct the synthesis of the 31K and 30K proteins, but the level of synthesis of these two proteins observed in gels of the translation products is approximately 10-fold less than the level observed when pMA-ACG1-selected mRNA is used. It is possible that the 31K and 30K proteins synthesized by pMA-ACG RNA and pMA-PB RNA are different proteins which fortuitously comigrate. However, this would be an unusual coincidence, and we are investigating this phenomenon since it may reflect a novel transcriptional phenomenon.

RNA selected by two clones, pMA-DA1 and pMA-RJ1, did not direct the synthesis of any observable proteins under the conditions used. It is possible that these regions encode very large proteins (>85K) which are not synthesized efficiently by the translation system. However, there are many other explanations for these negative results, including the possibility that the homologous mRNAs are present in very low concentrations in the late mRNA preparation.

Three clones, pMA-B1E1, pMA-DO1, and pMA-B2H1, direct the synthesis of proteins that are not found in NOV, as judged by a lack of comigration in gels. We cannot categorically state that these are nonstructural proteins since it is possible that the proteins are processed in vivo (1). For instance, the 61K protein of pMA-B1E1 may be related to the NOV 64K to 65K structural protein, which is somewhat diffuse and may be glycosylated in vivo. Both the 56K protein of pMA-DO1 and the 37K protein of pMA-B2H1 migrate in close proximity to NOV strutural proteins. Post-translational processing of AcNPV proteins in vivo resulting in major size alterations of the proteins does not appear to be an extensive phenomenon (2, 4, 25). Eventually, genetic mutants and marker rescue may be necessary to confirm specific protein assignments. The pMA-CD1-selected RNA directs the

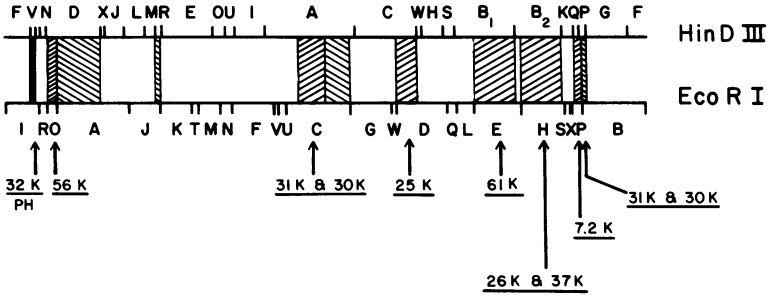

FIG. 8. Summary of cDNA map positions and selected proteins. The physical map of AcNPV is presented in linear form. The sizes of the proteins translated from AcNPV cDNA-selected RNA are indicated.

synthesis of a 25K protein which comigrates with a structural protein of NOV. In all cases of comigration of proteins, the physical identity or antigenic similarity of the proteins must be established before it can be concluded that the proteins are indeed related.

The 11 different regions of DNA delineated by the cDNAs isolated in this work are dispersed throughout the viral genome. A summary of the map positions of the cDNAs and the proteins synthesized from cDNA-selected RNAs is shown in Fig. 8. Transcriptional studies with these cDNA clones are under way and should reveal considerable information concerning the size(s) of the homologous transcript(s) and the temporal control of each of the transcripts. All of the cDNAs represent RNAs found late in infection, but this does not mean that these clones are found exclusively late in infection.

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